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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 15 August 2003 with an application for Letters Patent number 527607 made by AgResearch Limited.

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Dated 1 September 2004.



Neville Harris
Commissioner of Patents, Trade Marks and Designs



PATENTS FORM NO. 4

Appn Fee: \$50.00

James & Wells ref: 42247/29

PATENTS ACT 1953
PROVISIONAL SPECIFICATION

Channel Blocking Compounds

WE, AgResearch Limited, a New Zealand company of East Street, Ruakura Campus, Hamilton, New Zealand, do hereby declare this invention to be described in the following statement:

Intellectual Property
Office of NZ

15 AUG 2003

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CHANNEL BLOCKING COMPOUNDS

TECHNICAL FIELD

The present invention relates to compositions containing compounds of the group of alkaloid compounds termed lolitrem for use as an ion channel antagonist and 5 to a method of use of these compositions. More specifically, the lolitrem compounds are derived from the species *Neotyphodium* (*formally Acremonium*) *loli* and are used as potassium channel antagonists.

BACKGROUND ART

10 Ion channels are defined as transmembrane pores that present a hydrophilic channel for ions to cross a lipid bilayer down their electrochemical gradients. Some degree of ion specificity is usually observed and typically a million ions per second may flow. Channels may be open spontaneously, like the potassium leak channel, or they may be voltage-gated, like the voltage-gated sodium channel or 15 ligand-gated like the acetylcholine receptor.

Ion channels generally are the subject of much research to understand the roles they have in normal physiological systems and in disease states.

Potassium ion channels are selective for potassium ions. There are diverse types 20 of potassium ion channels with different functions, for example: delayed rectifier channels, M channels, A channels, inward rectifier channels, and calcium-activated potassium channels.

Large or high conductance calcium-activated potassium channels are also termed as BK channels, K_{Ca} , maxi-K. *Slowpoke* (*slo*) is the name of the gene that encodes the pore-forming α subunit of the channel, e.g. *hSlo* – human gene, *mSlo* –

mouse, *dSlo* drosophila – fruit fly. Accessory β subunits ($\beta 1-\beta 4$) associate with the α subunit to generate BK channel diversity. High conductance (BK) channels are gated by Ca^{++} and membrane potential with a unit conductance of 100 to 300 picoSiemens (pS)

- 5 Calcium-activated potassium channels also include intermediate conductance (IK) and small conductance (SK) channels. IK potassium channels are more sensitive to Ca^{++} than BK channels and are gated only by internal Ca^{++} ions, having a unit conductance of 25 to 100 pS. SK channels are also highly sensitive to Ca^{++} and have minimal voltage sensitivity, and a unit conductance of 2 to 25 pS.
- 10 For the purposes of this specification, the term BK channel or potassium channel will be referred to although this should not be seen as limiting.

BK channels are expressed in many tissues and regulate important physiological functions. They are activated in response to depolarising voltages and to increased intracellular calcium. Their activation results in efflux of potassium ions 15 causing hyperpolarisation which dampens cellular excitability. BK channels are expressed in most tissues and control a large variety of physiological processes including smooth muscle tone, neurosecretion and hearing.

- In blood vessels, BK channels oppose vasoconstriction, allowing vasorelaxation and thereby regulate arterial tone (i.e. blood pressure) (Brenner, Perez et al 2000).
20 In the brain, they reduce excessive neurotransmitter release. They are also expressed in the cochlea of the ear where they have a specialised role in frequency tuning of hair cells, acting in concert with other ion channels (Gribkoff, et al 2001; Orio et al 2002).

BK channels are also expressed in other tissues where their role is not known, e.g.
25 ovary, testis and kidney (Brenner, Jegla et al 2000).

Compounds that block (inhibit) a biologic activity or process in this transfer of ions across a cell membrane are called 'blockers'. They may also be termed antagonist compounds as the compounds reduce or prevent ion transfer. For the purposes of this specification the term antagonist will be used however this should not be seen
5 as limiting.

Known marketed drugs that block potassium channels include GlyburideTM, GlipizideTM and TolbutamideTM. Other naturally occurring toxins that are known to block potassium channels include Apamin, Iberiotoxin, Charybdotoxin, Noxiustoxin and Kallotoxin. US 5,541,208 describes uses of these blockers and the use of
10 paxilline, a further blocking compound, and is incorporated herein by reference.

Lolitrem belongs to the broader group of alkaloid compounds incorporating indole diterpenes.

Assay techniques for identifying lolitrem compounds are known, for example see NZ 236879.

15 Lolitrem compounds are present in perennial ryegrass (*Lolium perenne*) infected with the endophytic fungus *Neotyphodium* (formally *Acremonium*) *loli*.

Endophytes are symbiotic fungi and are prevalent in at least New Zealand pastures. The fungal metabolites from these endophytes are thought to serve as chemical defence systems for the fungi that produce them. They may also be of
20 use in protecting the food source from consumption by other organisms (US 4,973,601).

The lolitrem are neurotoxic indole-diterpenes and are the principal causative agents of ryegrass staggers. This is a condition in which animals grazing on endophyte infected ryegrass-dominant pastures develop ataxia, tremors, and
25 hypersensitivity to external stimuli. The lolitrem neurotoxin (staggers) reaction is

long acting but is however completely reversible (Smith et al 1997, McLeay et al 1999). The time course of tremors induced by lolitrem B is dramatically different from that of other indole diterpenes, for example paxilline and analogues. When injected into mice paxilline analogues induce tremors of rapid onset and short duration while tremors induced by lolitrem derivatives take hours to reach maximum intensity and last for days.

Whilst at least some lolitrems and other indole diterpenes, for example paxilline, are known to cause tremogenicity (tremogenic mycotoxins) there is no proven link between tremogenicity and BK channel blocking.

10 Some linkage is inferred between tremogenicity and neurotransmitter release (Mantle 1983, Gallagher et al 1986, Smith et al 1997, McLeay et al 1999, Wang et al 2003).

Some alkaloid compounds and more specifically indole diterpenes, block BK channels (e.g. paxilline, US5,541,208) and some do not. The alkaloids that inhibit 15 BK channels include both tremorgens and non-tremorgens. Structural moieties that are important for BK channel antagonism have been determined for some paxilline derivatives (Knaus et al 1994). However, for other types of indole diterpenes (e.g. lolitrems) whether a given compound will inhibit the BK channel cannot yet be predicted from structure alone but must be determined empirically.

20 Within a given structural class of indole diterpene, tremogenicity cannot be predicted by structure. For example, while paxilline and lolitrem B are tremogenic, lolilline, which is intermediate in structure between the two, is non-tremogenic.

The structural features required for tremogenicity are also different for each group of structurally related indole diterpenoid compound. An acetal-linked isoprene unit, 25 the presence of A/B rings and the stereochemistry at the A/B ring junction have been identified as important structural features for the tremogenicity of lolitrem

derivatives. Different structural features are required for tremogenicity for other Indole diterpene compounds.

As channel blockers have a variety of pharmaceutical uses and have been found to be beneficial for treatment of some diseases (for example Parkinson's disease, 5 US 5,541,208), such blocking compounds are of interest, particularly in the development of new therapies.

It is an object of the present invention to provide an alternative channel blocking compound or at least to provide the public with a useful choice.

All references, including any patents or patent applications cited in this 10 specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not 15 constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

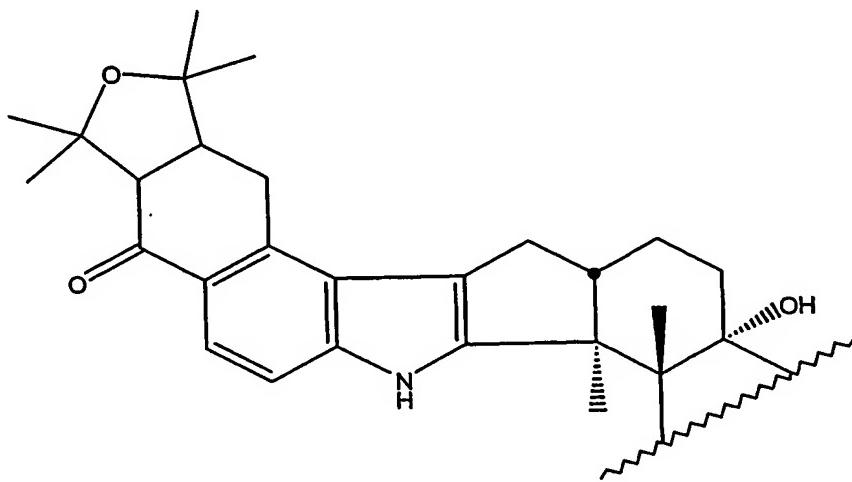
It is acknowledged that the term 'comprise' may, under varying jurisdictions, be attributed with either an exclusive or an inclusive meaning. For the purpose of this 20 specification, and unless otherwise noted, the term 'comprise' shall have an inclusive meaning - i.e. that it will be taken to mean an inclusion of not only the listed components it directly references, but also other non-specified components or elements. This rationale will also be used when the term 'comprised' or 'comprising' is used in relation to one or more steps in a method or process.

Further aspects and advantages of the present invention will become apparent 25 from the ensuing description which is given by way of example only.

DISCLOSURE OF THE INVENTION

It has been found by the applicant that lolitrem compounds are antagonists of potassium channels.

- 5 According to one aspect of the present invention there is provided a composition that contains a pharmacologically effective amount of at least one ion channel antagonist compound with the structure (I):



STRUCTURE (I)

- 10 or derivatives thereof.

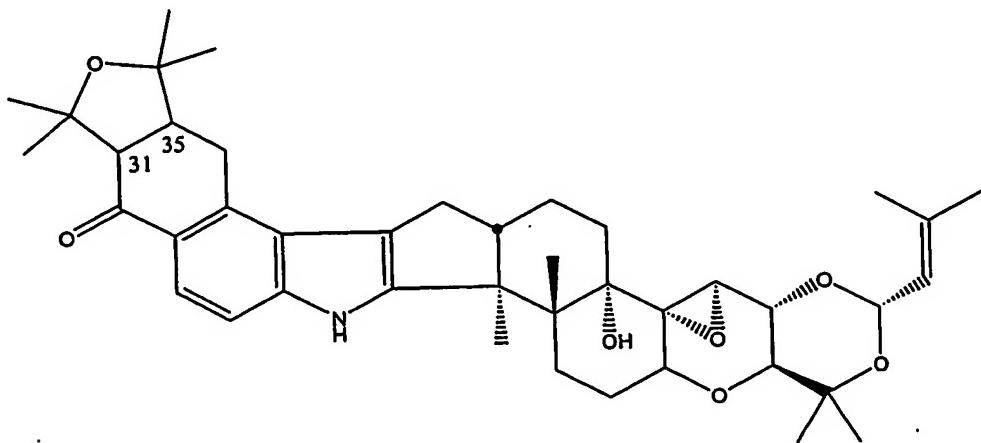
Preferably the composition, further includes pharmaceutically and physiologically acceptable carriers.

Preferably, derivatives of structure (I) are selected from the group consisting of: salts, analogues, isomers, and combinations thereof.

- 15 Preferably, the antagonist compound is selected from the group consisting of: lolitrem B, lolitrem A, lolitrem F, 31-epi lolitrem F, 31-epi lolitrem B, lolitrem E,

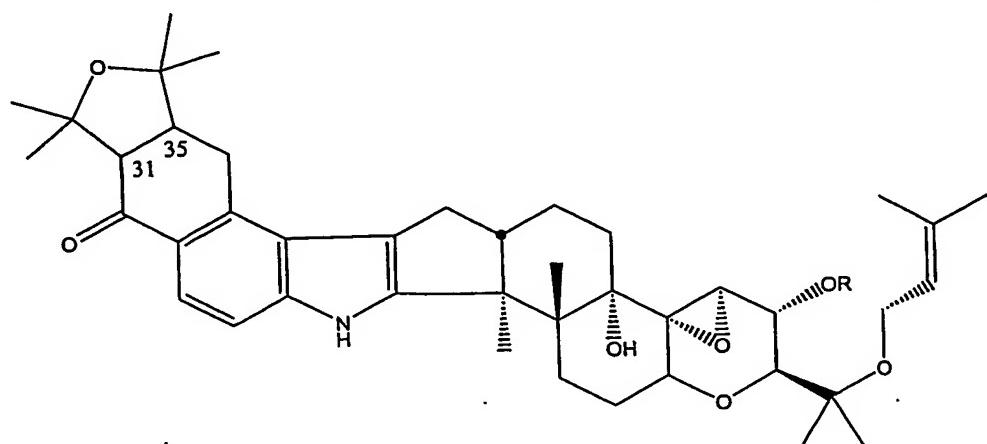
lolitrem E acetate, lolitrem L, lolitrem G, lolitrem C, lolitrem M, lolitriol, lolitriol acetate, lolitrem N, lolitrem J, lolitrem H, lolitrem K, lolicine A and B, 30-desoxy lolitrem B-30a-01, 30-desoxy-31-epilolitrem B-30a-01, 30-desoxylolitrem B-30-ene loliline and combinations thereof.

- 5 Preferably, the antagonist compound is structure (II):



STRUCTURE (II)

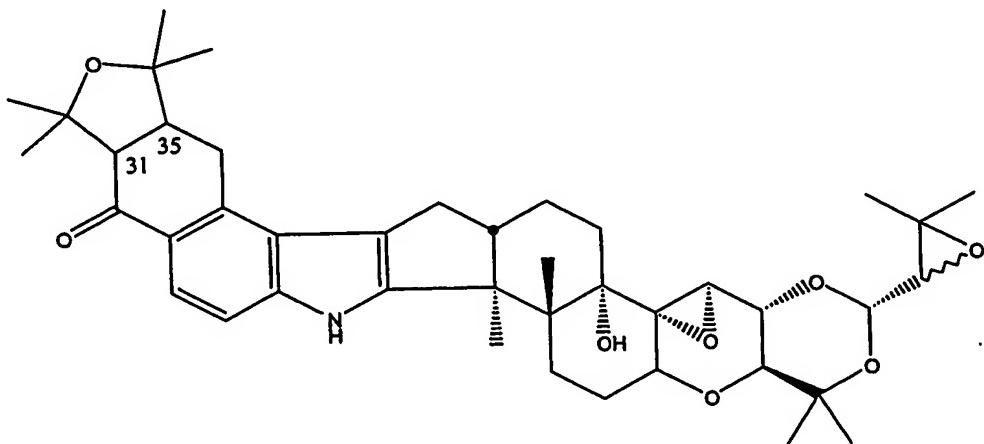
- which includes compounds selected from the group consisting of: lolitrem B = 31 α , 35 β stereochemistry; 31-epilolitrem B = 31 β , 35 β stereochemistry; lolitrem F = 31 α , 35 α ; 31-epilolitrem F = 31 β , 35 α .
- 10 Preferably, the antagonist compound is structure (III):



STRUCTURE (III)

which includes compounds selected from the group consisting of: lolitrem E = 31α , 35β stereochemistry where R = H or acetate; lolitrem L = 31α , 35α stereochemistry where R = H or acetate.

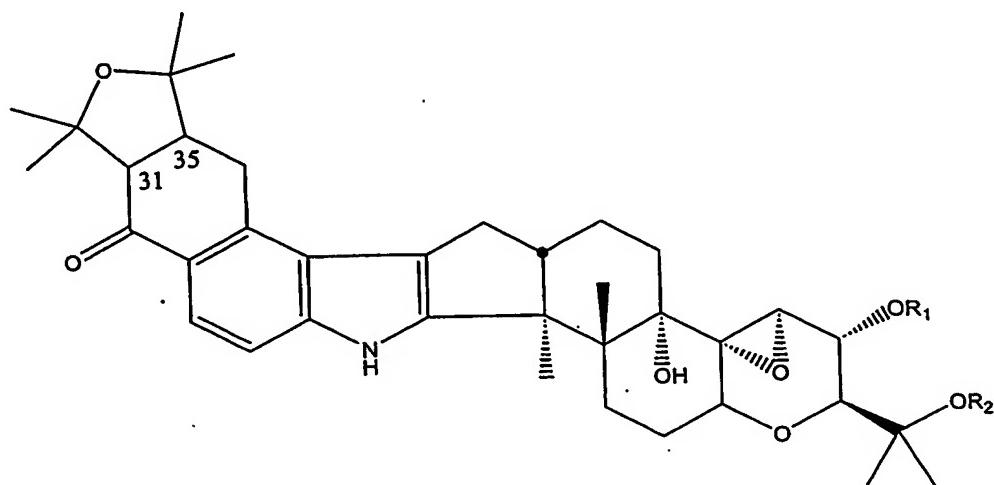
- 5 Preferably, the antagonist compound is structure (IV):



STRUCTURE (IV)

which includes compounds selected from the group consisting of: lolitrem A = 31α , 35β stereochemistry; lolitrem G = 31α , 35α stereochemistry.

- 10 Preferably, the antagonist compound is structure (V):

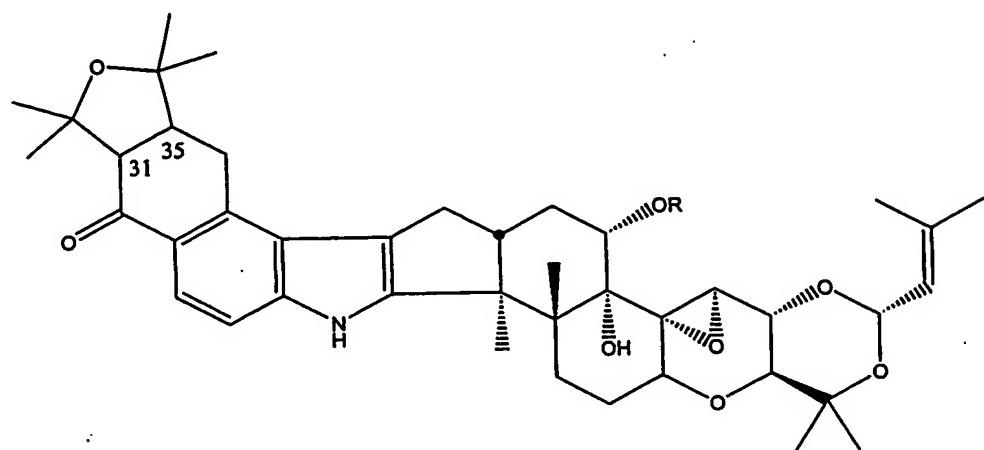


STRUCTURE (V)

which includes compounds selected from the group consisting of: lolitriol; = 31 α , 35 β stereochemistry where R₁ = H or acetate and R₂ = H; lolitrem N = 31 α , 35 α stereochemistry where R₁=H or acetate and R₂=H; Lolitrem J = 31 α , 35 β stereochemistry where R₁ = H or acetate and R₂ = acetate.

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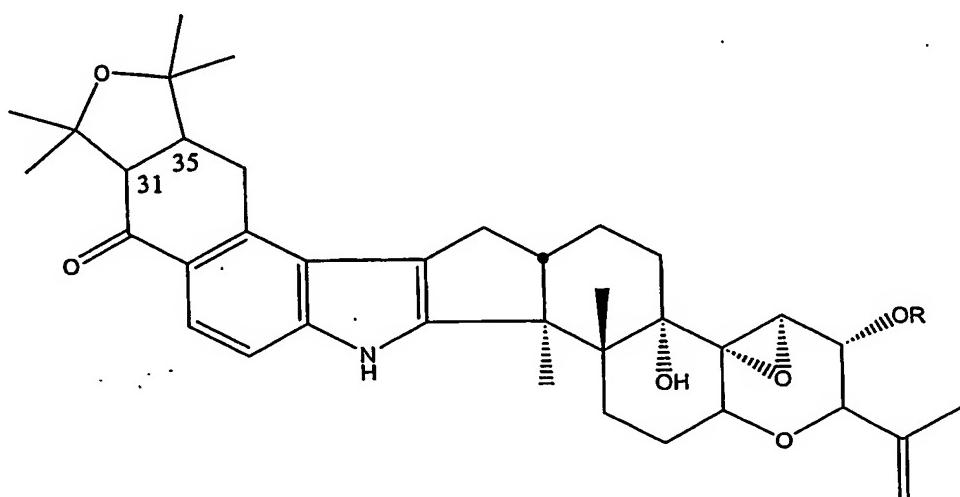
Preferably, the antagonist compound is structure (VI):



STRUCTURE (VI)

which includes lolitrem H = 31 α , 35 β stereochemistry where R = H or acetate.

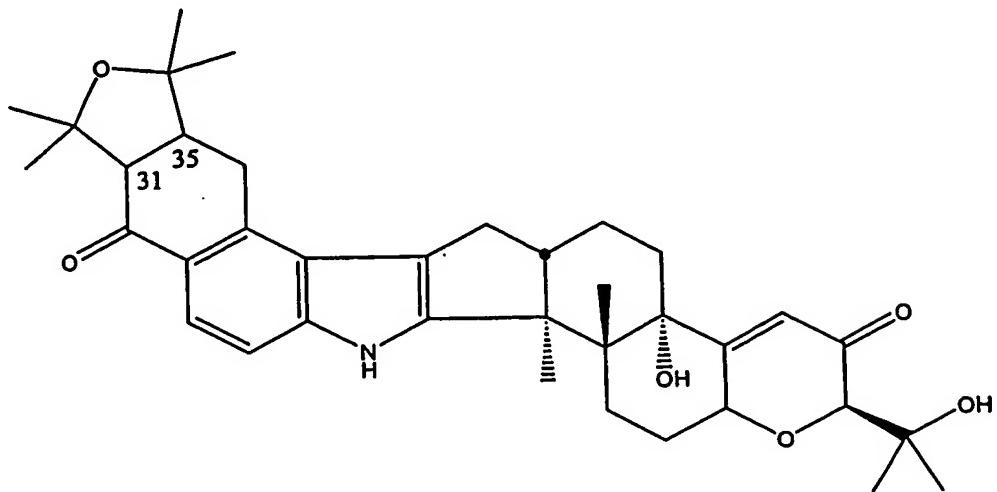
10 Preferably, the antagonist compound is structure (VII):



STRUCTURE (VII)

which includes lolitrem K = $31\alpha, 35\beta$ stereochemistry, where R = H or acetate.

Preferably, the antagonist compound is structure (VIII):

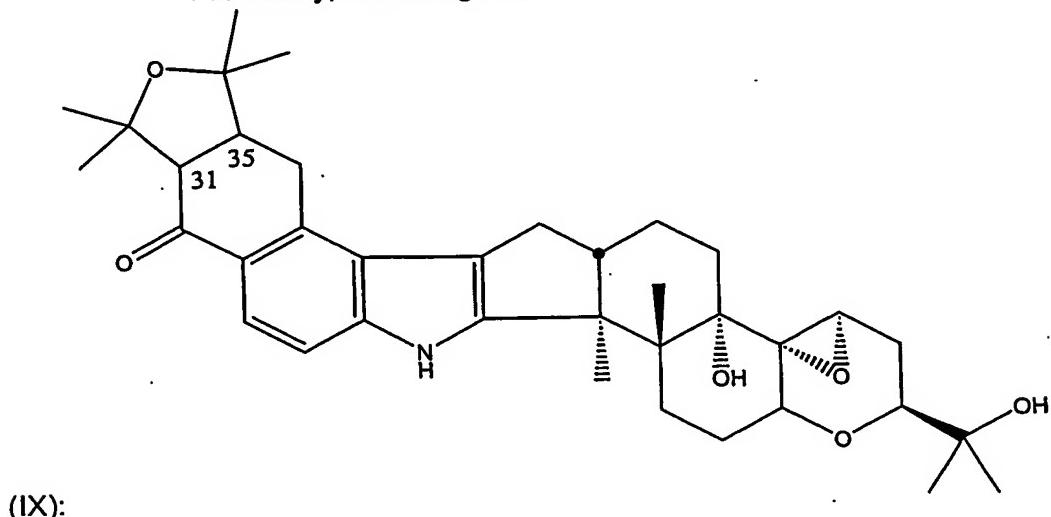


STRUCTURE (VIII)

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which includes lolilline = $31\alpha, 35\beta$ stereochemistry.

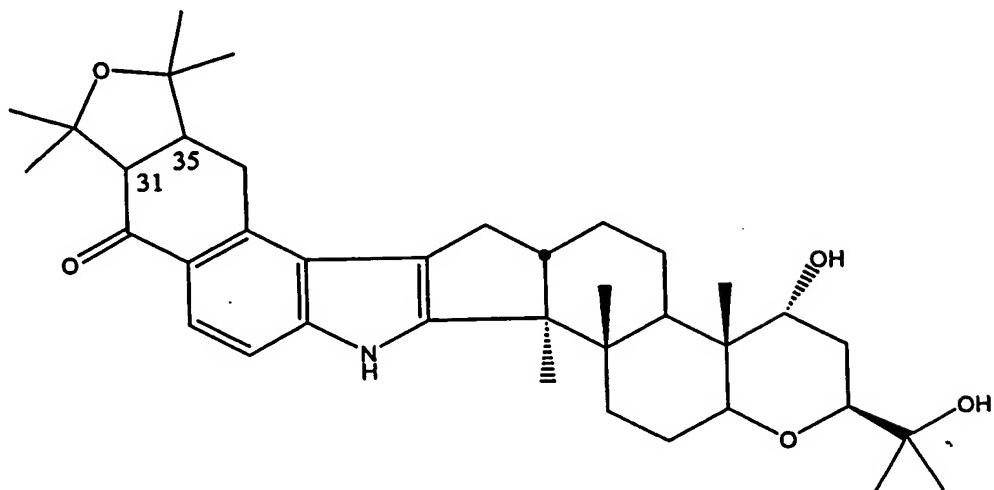
Preferably, the antagonist compound is structure



STRUCTURE (IX)

which includes lollitrem M = $31\alpha, 35\beta$ stereochemistry.

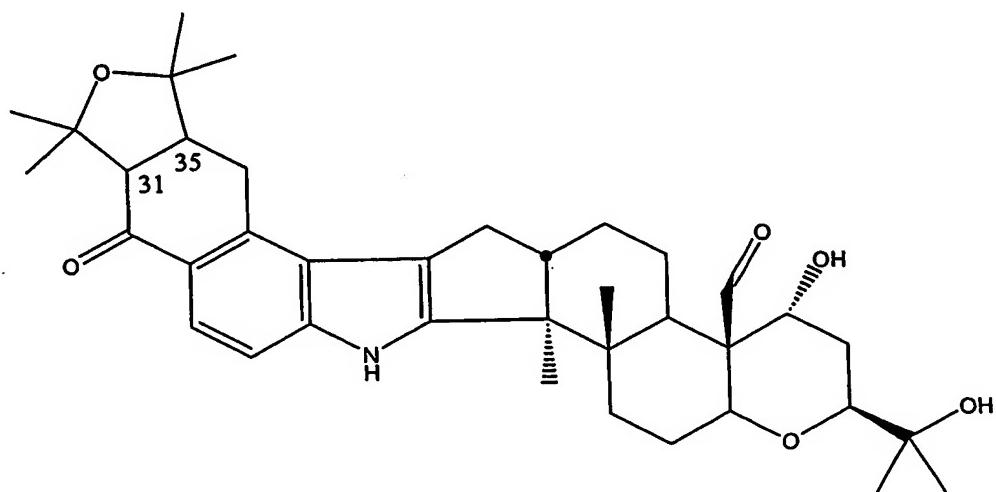
Preferably, the antagonist compound is structure (X):



STRUCTURE (X)

5 which includes lolicine A = $31\alpha, 35\beta$ stereochemistry.

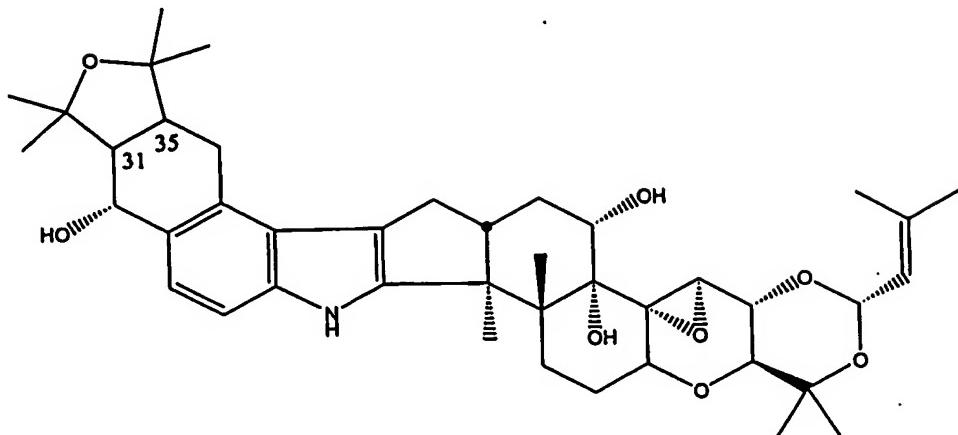
Preferably, the antagonist compound is structure (XI):



STRUCTURE (XI)

which includes lolicine B = $31\alpha, 35\beta$ stereochemistry.

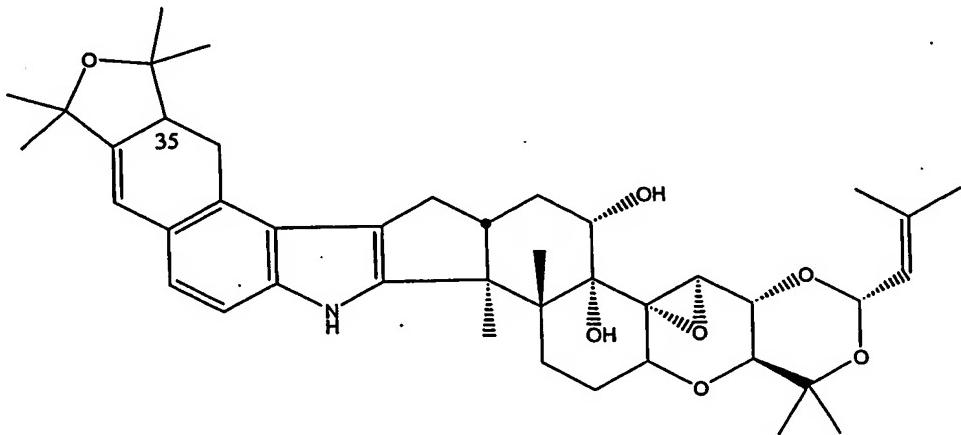
Preferably, the antagonist compound is structure (XII):



STRUCTURE (XII)

- which includes compounds selected from the group consisting of: 30-
 5 desoxylolitrem B-30 α -ol = 31 α , 35 β stereochemistry; 30-desoxy-31-epiolitrem B-
 30 α -ol = 31 β , 35 β stereochemistry.

Preferably, the antagonist compound is structure (XIII):



STRUCTURE (XIII)

- 10 which includes 30-desoxylolitrem B-30-ene = 35 β stereochemistry.

Preferably, the lolitrem compounds are extracted from endophyte-infected

perennial ryegrass seed (*Lolium perenne*). Derivatives may be synthetically prepared from these compounds.

Preferably, the potassium channel is a large conductance calcium activated potassium (BK) channel although embodiments including intermediate 5 conductance (IK) and small conductance (SK) are also incorporated herein.

Preferably, for lolitrem B, the degree of antagonist inhibition is $97.9\% \pm 0.5\%$ (n=4) for a composition containing approximately 20nM lolitrem B. The half maximal degree of antagonist inhibition is found for a composition containing approximately 2.9nM of lolitrem.

10 It is the applicants understanding that the above levels of antagonist behaviour found in *in vitro* experiments indicates that lolitrem compounds have a high apparent affinity for at least *hS/o* channels. That is, the lolitrem compounds reduce and inhibit potassium currents through *hS/o* channels.

15 The antagonist effect of the lolitrem B composition is not able to be reversed by wash out. It is applicant's experience that this effect is for concentrations of 10nM or greater of lolitrem B compound.

According to a further aspect of the present invention there is provided a method 20 of preventing repolarisation or hyperpolarisation of a cell, wherein the cell contains an ion channel, including the administration to the cell of a pharmacologically effective amount of an ion channel antagonist substantially as described above.

According to a further aspect of the present invention there is provided the use of a composition substantially as described above for preventing repolarisation or hyperpolarisation of a cell, wherein the cell contains an ion channel.

The above description shows that there is provided a composition that has an 25 antagonist effect on ion channels. The composition can be used in methods for

blocking ion channels including BK channel blocking. One embodiment envisaged is that of a diagnostic pharmaceutical for blocking such channels.

BRIEF DESCRIPTION OF DRAWINGS

5 Further aspects of the present invention will become apparent from the following description which is given by way of example only and with reference to the accompanying drawings in which:

- 10 Figure 1 shows two current recordings (A and B) for different concentrations of lolitrem B compared to control recordings of (A) ramp potential and (B) depolarising voltage pulses to +150 mV to determine the degree of antagonist inhibition; and,
- Figure 2 shows a graph comparing the degree of antagonist inhibition observed against lolitrem B concentration.

15 BEST MODES FOR CARRYING OUT THE INVENTION

The results found from experiments carried out by the applicant are now described.

Experiment 1

- 20 In this experiment, *hS/o α* subunit large conductance calcium-activated potassium (BK) channels with an N-terminal c-myc tag in the mammalian vector pcDNA (Meera *et al*, 1997) were transiently expressed in human embryonic kidney cells (cell type HEK293).

Cell Culture Preparation

Human embryonic kidney cells were grown in a mix of DMEM (Dulbecco's Modified Eagle Medium, GibcoBRL Cat#12100-038) and 2.5 mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), supplemented with minimal essential amino acids and 10 % fetal bovine serum.

Cells were subsequently plated into 24-well plates, grown to 95% confluency and transfected 24 hours later with 10 µg of *hSlo* and 2 µg CD4 (pcDNA) and 2 µL Lipofectamine 2000TM.

10 Cells were plated onto cover slips 24 hours later. CD4 antibody-labelled beads were used to identify transfected cells. Macroscopic currents were recorded from excised inside-out patches at 3 days post-transfection.

Lolitrem B preparation

15 Lolitrem B was extracted from perennial ryegrass seed infected with *Neotyphodium lolii*.

A stock of 100 µM Lolitrem B was made up in dimethyl sulfoxide (DMSO). This was diluted to the appropriate concentration in electrophysiological solutions. The final DMSO concentration was 0.1 % for 100 nM lolitrem B and did not exceed 0.02
20 % for lower concentrations.

Electrophysiology

Solutions

The bath solution that was applied to the internal side of the cell membrane of the inside-out membrane patch was (mM): 140 KMeSO₃, 2 KCl, 20 HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), 5 HEDTA (N-(2-hydroxyethyl)ethylene-diaminetetraacetic acid) and 3.65 CaCl₂ to give 10 µM free calcium with a pH of 7.2.

The pipette solution applied to the external side of the cell membrane of the inside-out membrane patch was (mM): 140 KMeSO₃, 2 KCl, 20 HEPES, 2 MgCl₂, with a pH of 7.2.

Macroscopic currents were recorded in an inside-out patch-clamp configuration using an amplifier, interface and data collection software. Data were filtered at 5 kHz and sampled at 20 µs intervals. Fast capacitance compensation was used to cancel the fast transient. Leak subtraction was used although the background potassium current was small.

Results

The effect of Ioltrem B on potassium currents in excised inside-out patches from cells expressing hSlo BK channels is shown in Figure 1. The Figure shows two current recordings (A and B) for different concentrations of Ioltrem B compared to control recordings of (A) ramp potential and (B) depolarising voltage pulses to +150 mV to determine the degree of antagonist inhibition.

In Figure 2, solid circles show the mean normalised current (fraction blocked) in three or more cells and the vertical bars are mean ± 1 S.E.M. The curve is a fit of the Hill equation to the data.

Figure 2 shows that the application of 20 nM lolitrem B to the perfusion bath,
5 resulted in complete inhibition of the BK channel current. The level of antagonist
inhibition was less at lower lolitrem B concentrations.

Channels were activated by voltage pulses to +150 mV every minute in the
presence of 10 μ M free calcium. Control current responses were recorded over 5
minutes. Only patches that remained stable over this time were used in
10 experiments.

The current block produced by 10 nM or greater lolitrem B could not be reversed,
even after wash-out with control solution for 30 minutes at a flow rate of 4ml/min in
three experiments.

At 2 nM lolitrem B partial inhibition was observed.

15 Dose-response experiments were carried out to determine the concentration
range over which lolitrem B was effective.

Recovery from inhibition could not be used to validate reductions in current at
different lolitrem B concentrations as being due to the presence of drug, nor could
different concentrations be applied in random order. However, by applying
20 cumulative doses of lolitrem B to the same membrane patch, it was found that
increases in the degree of current block with increased lolitrem B concentration
were consistent between cells.

Each concentration of lolitrem B was applied for 10 minutes and fractional block
calculated as the decrease in current as a fraction of the control. The current
25 amplitude was the mean current over the last half of the voltage pulse to +150 mV.

The data was analysed and fitted using the Hill equation which gave an estimate of the concentration of half maximal inhibition (IC_{50}) of 2.9 ± 1.2 nM from 11 cells.

- The concentration range of inhibition observed for lolitrem B is similar to that reported for other indole diterpenes including: paxilline, aflatrem, penitrem A, 5 paspalinine, paspalitrem A and C, verruculogen and paspalinine applied to either native or heterologously expressed BK channels (Knaus et al., 1994, Sanchez and McManus, 1996, Gribkoff et al., 1996).

- Difficulty in reversing channel block is also noted for these compounds, although paxilline block by low concentrations could be partially reversed by washout 10 (Knaus et al., 1994).

Thus it can be seen from the above experiment that at least lolitrem B has a blocking effect on at least BK channels.

Experiment 2

- 15 In this experiment, it is shown that whilst some lolitrem compounds are known to cause tremogenicity to one extent or another, it is not certain that there is a direct link to BK channel blocking and vice versa.

The experiment uses 31-epi-lolitrem B, a known non-tremogenic lolitrem compound as described in Munday-Finch et al 1996.

- 20 The same methods were used for testing the antagonist effect of 31-epi-lolitrem B as described in Experiment 1 above.

The results found showed that 31-epi-lolitrem B at a concentration of 100 nM inhibits BK channel currents (α subunit) by $88.3\% \pm 4.7\%$ ($n=3$).

The above result shows that a non-tremogenic compound can inhibit BK channels. Tremogenicity is thus unlikely to be directly linked to BK channel blocking which is a similar result to that found in general Indole diterpene studies (Knaus et al 1994).

- 5 Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope thereof.

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AgResearch Limited
by their Attorneys

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